The Chemical and Biochemical Properties of Fluorocitric Acid

By P. F. V. WARD and R. A. PETERS*
A.R.C. Institute of Animal Physiology (Biochemistry Department), Babraham, Cambridge

(*Received* 18 *July* 1960)

Two methods have been devised for the preparation of fluorocitric acid (1-fluoro-2-hydroxypropane-1:2:3-tricarboxylic acid), the one a biosynthetic method (Peters, Wakelin & Buffa, 1953) and the other a synthetic method (Rivett, 1953). In the biosynthetic method the substance is formed from fluoroacetate by living tissues and in the synthetic method it is formed from fluoroacetate chemically by a method based on a synthesis of citric acid (Lawrence, 1897). No alternative route for the chemical synthesis of fluorocitric acid is available. Fluorocitric acid has been used widely as an enzyme inhibitor despite the fact that the biosynthetic acid differs greatly in its action upon aconitase from the synthetic acid (Morrison & Peters, 1954). It has recently been discovered (Ward & Peters, 1957) that what were thought to be pure samples of synthetic fluorocitric acid could be resolved chromatographically into a large number of components. The object of the present work has been to investigate the properties of some of these components in greater detail and to compare their biochemical activity with biosynthetic fluorocitric acid.

EXPERIMENTAL

Materials. Samples of triethyl fluorocitrate were prepared by Dr E. M. Gal and Dr N. F. Taylor. Dr E. M. Gal also prepared fluorocitric acid containing ^{14}C (1-fluoro-2-hydroxy-[3-^{14}C]-propane-1:2:3-tricarboxylic acid). The barium salt of fluorocitric acid was prepared by Dr D. E. A. Rivett. Trisodium fluorocitrate was prepared by Dr J. Walker. All the above-mentioned samples were synthesized by the method of Rivett (1953). Fluorocitric acid was prepared biosynthetically from rabbits by Peters *et al.* (1953). Silicic acid (Mallinckrodt Chemical Works) was obtained from Savory and Moore Ltd. Solvents for paper and column chromatography were of AnalaR grade.

Paper chromatography. This was done on Whatman no. 1 paper in glass tanks by upward solvent development. The solvent was propan-1-ol–aq. ammonia soln. (sp.gr. 0·880)–water (6:3:1, by vol.). A solution of fluorocitric acid (10 μl. containing 100 μg.) was spotted on the paper and components were detected by the use of indicator sprays according to the method of Reid & Lederer (1951).

Column chromatography. Ion-exchange chromatography was carried out according to the method of Peters *et al.* (1953) on the ^{14}C-labelled sample of fluorocitric acid prepared by Dr E. M. Gal. Samples of the acid (50 mg.) were chromatographed with 0·02 N-HCl as an eluent. The eluate was collected in 5 ml. fractions and the peaks were detected by pipetting and drying 0·1 ml. samples from each fraction on aluminium planchets. The radioactivity of the samples was measured with an end-window Geiger counter. Self-absorption was negligible provided that the quantity of material on the planchets did not exceed 0·25 μmole of fluorocitric acid, or similar amounts of other components.

Silicic acid-column chromatography was used according to the procedure of Bulen, Varner & Burrell (1952), except that 2·2 ml. fractions were collected. The peaks were detected by titration of appropriate fractions with 0·1 N-NaOH. This was done directly in the collecting tubes by adding 1 ml. of CO_2-free water and 1 drop of phenol red and using a standard Conway burette, to the outlet of which had been attached a glass tube drawn to a fine capillary at its end. Air free of CO_2 was bubbled through the contents of the test tubes during titration to facilitate intimate contact between the two phases without producing an emulsion. Radioactive acidic components were detected as described above as well as by titration.

Preparation of samples for chromatography. Both synthetic and biosynthetic samples of fluorocitric acid were chromatographed as free acid. The sodium salt was converted into the free acid by passing a solution of it down an Amberlite IR-120 column, which was washed with water and the combined effluents were evaporated (Ward, Gal & Peters, 1956). Triethyl fluorocitrate was hydrolysed under both alkaline and acid conditions (see below).

Estimation of biological activity of fluorocitric acid

Tests were made on (*a*) guinea-pig-kidney particles with citrate as substrate, or (*b*) pigeon-brain particles with pyruvate and fumarate as substrate, or (*c*) isolated soluble aconitase.

Kidney-particle test. This was done essentially as described previously (Peters & Wakelin, 1957) but in the later tests the kidneys were treated for 1·5 min. in a Potter homogenizer instead of grinding them in a mortar. Too long a period of homogenization gave a preparation in which the aconitase, even though it was still very active, was little inhibited by fluorocitrate. Poor inhibitions by fluorocitrate also occurred if more than 1 μmole of citrate was left after the 30 min. shaking at 38°. The particles prepared from a pair of kidneys (dry wt. approx. 50 mg.) were suspended in a mixture of 1% (w/v) KCl solution and 0·1 M-KH_2PO_4 neutralized to pH 7·2 with NaOH. Inhibition of citrate metabolism in the particles was induced by the fluoro acids and measured as follows. A mixture of kidney-particle suspension (1·9 ml.), 0·8% $MgCl_2,6H_2O$ solution (0·1 ml.), adenosine triphosphate (0·33 mg. of disodium salt in

* Present address: Department of Biochemistry, University of Cambridge.

0·1 ml.), trisodium citrate (10 μmoles in 0·4 ml.), fluoro acid as sodium salt in 0·1 ml. and 1% (w/v) KCl solution (0·3 ml.), was incubated for 30 min. at 38° in air. After incubation, 25% (w/v) trichloroacetic acid solution (1·0 ml.) was added, and solid material was centrifuged or filtered off. Citrate was estimated in the supernatant solution by the method of Taylor (1953). A control experiment was run with 1% (w/v) KCl solution in place of the fluoro acid solution and served as a standard by which the inhibition of the utilization of citrate caused by the fluoro acids could be measured.

Brain-particle test. This was done as described by Peters & Wakelin (1957). It was used to distinguish between fluorocitrate and fluoroacetate when they are present in the same solution; the brain particles are insensitive to fluoroacetate, hence the relative amounts of the two substances can be measured by comparing the inhibition obtained in kidney particles with that obtained in brain particles.

Isolated aconitase test. This test was carried out essentially as described by Morrison & Peters (1954), at first with purified aconitase prepared by Dr J. F. Morrison, and later with an aconitase preparation taken to the first ethanol precipitate stage as described by Morrison (1954). The cuvettes contained 2-amino-2-hydroxymethylpropane-1:3-diol (tris)–HCl (0·05 M) buffer, pH 7·4 (0·3 ml.), enzyme (0·1–0·2 ml.), neutralized inhibitor and water to 2·75 ml. After standing for 10 min. sodium (±)-*iso*citrate (2·76 μmoles in 0·01 ml.) was added from a micrometer syringe and the rate of formation of *cis*-aconitate was determined by measuring the increase in extinction of the solution at 240 mμ every 0·5 min. from 0·5 min. to 4 min. after addition. Under these conditions the maximum inhibition of the conversion of *iso*citrate into *cis*-aconitate by fluorocitrate is not more than 69%, irrespective of the amount of fluorocitric acid added. The tests were made against a control containing no inhibitor. In tris buffer and without addition of Fe^{2+} ions and cysteine, this preparation of aconitase is much more sensitive to fluorocitrate (Peters 1959, 1961), giving 50% maximum inhibition at 10^{-8}–10^{-9} M). The enzyme used contains about 50% still combined with Fe^{2+} ions.

RESULTS

Chromatography of fluorocitric acid. Paper chromatography of specimens of biosynthetic fluorocitric acid in the prepared ammonia solvent gave a single acidic spot with the same R_F (0·25) as citric acid but synthetic samples of fluorocitric acid under the same conditions gave as many as four acidic spots varying from R_F 0·25 to 0·65.

Ion-exchange-column chromatography as described gave only partial resolution of the sample of synthetic radioactive fluorocitric acid (1-fluoro-2-hydroxy-[3-^{14}C]-propane-1:2:3-tricarboxylic acid) into eight diffuse peaks. Paper chromatography of these peaks showed further resolution. Some of the peaks gave as many as three acidic spots on the paper, there being a gradual change in the relative intensity of the three spots from the first to the eighth peak. A radioautograph of the paper chromatogram showed that some of the strongly acidic spots were not radioactive.

Silicic acid-column chromatography gave a good separation of many of the components in samples of fluorocitric acid and the results with any one sample were reproducible. Fig. 1 illustrates the chromatography by this method.

Analysis of samples of fluorocitric acid by silicic acid chromatography. Fig. 1 (*a*, *b* and *c*) show the analysis of three separately synthesized samples. They were transformed into the free acid before chromatography. In sample (*a*) each mole of ester was hydrolysed by 4·5 g.-equiv. of NaOH at a concentration of 0·1 N at 20° for 24 hr., and in

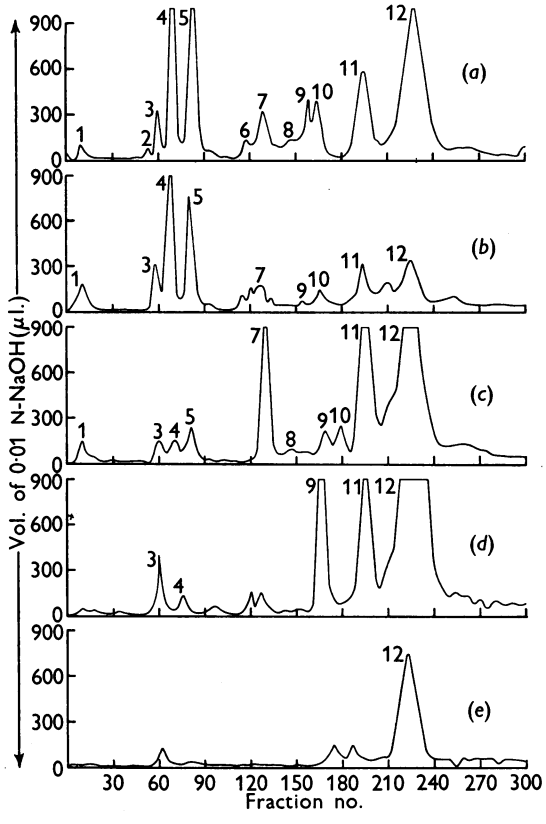

Fig. 1. Silicic acid-column chromatography of samples of fluorocitric acid. Sample (*a*): synthetic triethyl fluorocitrate hydrolysed by 0·1 N-NaOH at 20° for 24 hr. Sample (*b*): a second synthetic triethyl fluorocitrate hydrolysed by 0·1 N-NaOH at 20° for 48 hr. Sample (*c*): a third synthetic triethyl fluorocitrate hydrolysed by N-NaOH at 20° for 48 hr. Sample (*d*): a fourth synthetic triethyl fluorocitrate hydrolysed by 3 N-HCl at 100° for 6 hr. and precipitated as the barium salt. Sample (*e*): biosynthetic fluorocitric acid. Fraction volumes were 2·2 ml. and eluting solvents were used as follows: fractions 1–48, butan-1-ol–chloroform (5:100, v/v); fractions 49–108, butan-1-ol–chloroform (15:100, v/v); fractions 109–157, butan-1-ol–chloroform (25:100, v/v); fractions 158–300, butan-1-ol–chloroform (35:100, v/v).

sample (*b*) by 4·0 g.-equiv. of NaOH at a concentration of 0·1 N at 20° for 48 hr. and in sample (*c*) by 6·0 g.-equiv. of NaOH at a concentration of 1 N at 20° for 48 hr. It can be seen that components 11 and 12 are larger and components 3 and 4 are smaller when the stronger alkali was used for hydrolysis. Sample (*d*) (Fig. 1) was given to us as the barium salt but was converted into the free acid before chromatography. It has been prepared from the ester by hydrolysis with 3 N-HCl at 100° for 6 hr. Components 3 and 4 are small and component 5 is missing, because unlike most of the others it forms a water-soluble barium salt and was separated in the preparation. The numbering of the components in Fig. 1 is identical with that in Figs. 2 and 4 and is used throughout the paper. In all these synthetic samples the chromatographic pattern is extremely similar below fraction 90 and above fraction 180 but the middle region between fractions 90 and 180 shows a variation with each individual sample. Sample (*e*) (Fig. 1) shows the chromatography of 17 mg. of fluorocitric acid prepared biosynthetically from rabbits treated with fluoroacetate. The major component is a peak at fraction 225, present also in all the synthetic samples. A number of the samples of synthetic fluorocitric acid contained citric acid as an impurity and this appears on the chromatograms as an extra component between components 11 and 12.

In Fig. 2 a comparison of the acid-containing peaks of the synthetic [^{14}C]fluorocitric acid with the radioactivity of the samples shows that the acidic component 5 is not radioactive. Three minor acidic components in the region of fraction 120 are also not radioactive but with the remainder there is a close correlation between acidity and radioactivity.

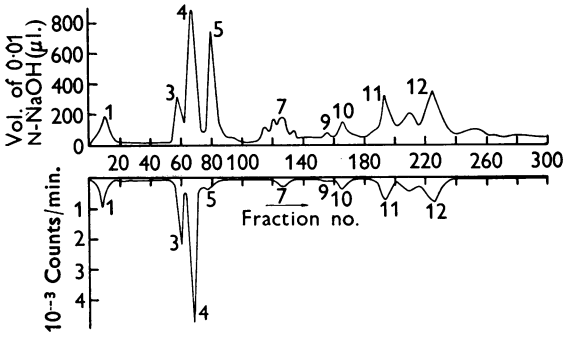

Fig. 2. Silicic acid-column chromatography of synthetic 1-fluoro-2-hydroxy-[3-^{14}C]-propane-1:2:3-tricarboxylic acid. Comparison of acidic and radioactive components. Fraction volumes were 2·2 ml. and eluting solvents were used as follows: fractions 1–48, butan-1-ol–chloroform (5:100, v/v); fractions 49–108, butan-1-ol–chloroform (15:100, v/v); fractions 109–157, butan-1-ol–chloroform (25:100, v/v); fractions 158–300, butan-1-ol–chloroform (35:100, v/v).

Biochemical activity of fluorocitric acid components. Of all the components eluted during silicic acid-column chromatography only component 12 in the various samples of fluorocitric acid showed marked biochemical activity. This component from all the samples caused decreased citrate metabolism in the guinea-pig-kidney test, increased the citrate level in the pigeon-brain test and inhibited isolated aconitase. Only on one occasion did any other component show appreciable biochemical activity. In this instance a component eluted around fraction 158 in Fig. 1 (*a*) showed an inhibition of isolated aconitase far greater than anything previously measured, but this could not be confirmed. The biochemical activity of component 12 varied according to the method of preparation of the sample. In biosynthetic preparations it was about twice as active on a weight basis as in synthetic ones. Table 1 and Fig. 3 illustrate this.

Effect of alkali on synthetic sodium fluorocitrate. It was noticed that during a period of 6 months one large sample of fluorocitric acid (Fig. 1*a*) stored as the sodium salt in a desiccator showed a change in both its chromatographic pattern and its biochemical properties. Components 3 and 4 had become larger and components 11 and 12 smaller while the whole sample had become less inhibitory to aconitase. When investigating this change it was discovered that although it could not be acceler-

Table 1. *Inhibition of citrate metabolism in guinea-pig-kidney particles by component 12 from synthetic and biosynthetic sources*

The particles were suspended in a mixture of 1% (w/v) KCl solution and 0·1 M-KH_2PO_4, neutralized to pH 7·2 with NaOH (Peters & Wakelin, 1957). To this suspension (1·9 ml.) was added 0·8% $MgCl_2,6H_2O$ solution (0·1 ml.), adenosine triphosphate (0·33 mg. of disodium salt in 0·1 ml.), trisodium citrate (10 μmoles in 0·4 ml.), inhibitor (2 μg. of sodium salt in 0·2 ml.) and 1% (w/v) KCl solution (0·3 ml.). After incubating for 30 min. at 38° in air 25% (w/v) trichloroacetic acid solution (1·0 ml.) was added and solid material centrifuged off. Citrate was estimated in the supernatant solution by the method of Taylor (1953). The control value was 0·4 μmole. Results are from one experiment in which the samples of fluorocitric acid were tested on the same kidney preparation. These results were supported by other experiments.

Inhibitor	Citrate remaining (μmoles)	Inhibition (%)
Biosynthetic 1	5·0	48
Biosynthetic 2	4·0	38
Synthetic 1	1·8	15
Synthetic 2	2·4	21

ated by treatment with acid it could be reversed by treatment with alkali (Table 2). This was the only sample that was kept for a period of years as the chromatographically heterogeneous sodium salt, and the separated components 11 and 12 from all samples of the synthetic acid showed no sign of spontaneous conversion into components 3 and 4, nor did isolated component 12 show any reduction in the inhibition of aconitase after storing.

The effect of alkali on components 3 and 4 of synthetic samples was as follows. Component 3 was transformed into component 11, which in turn was partially transformed into component 10. Similarly, component 4 was transformed into component 12, which was then partially transformed into component 7. The first set of transformations, that is from component 3 into 11 and 4 into 12, occurred under weaker alkaline conditions than the second set, in which component 11 changed to 10 and 12 to 7. This is illustrated in Fig. 4. For the maximum production of component 12, the component inhibitory to aconitase, the alkaline treatment must be carefully controlled, and from Table 3 the best conditions were 0·5N-NaOH at 20° for 24 hr. Even so, because the area of component 12 in this chromatogram was less than half the area of all the components, the actual amount of active fluorocitric acid was less than half the number of moles of acid present. These conditions also produced a sample with the maximum inhibition of aconitase (Fig. 3). For the optimum production of component 11 slightly less

Table 2. *Effect of alkali on the activity of synthetic sodium fluorocitrate and two of its chromatographic components towards citrate metabolism in guinea-pig-kidney particles*

Components 3 and 4 eluted during silicic acid chromatography of synthetic trisodium fluorocitrate and a sample of synthetic trisodium fluorocitrate of low biochemical activity were treated with alkali as indicated. Samples (2 μg.) of the treated substances were added to a preparation of guinea-pig-kidney particles. Figures are inhibition (%) after incubation for 30 min. at 38° in air.

Treatment	Inhibitor 1 (components 3 and 4)	Inhibitor 2 (synthetic trisodium fluorocitrate)
Nil	1	6
0·05N-NaOH at 54° for 1 hr.	—	10
0·5N-NaOH at 54° for 2 hr.	—	12
0·5N-NaOH at 20° for 1 hr.	—	9
0·5N-NaOH at 20° for 24 hr.	—	16
0·5N-NaOH at 50° for ⅔ hr.	15	—
0·5N-NaOH at 55° for 1 hr.	16	14

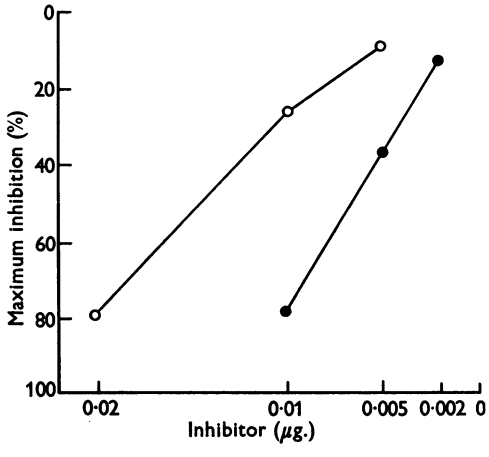

Fig. 3. Inhibition of isolated aconitase by biosynthetic and synthetic fluorocitric acid. A mixture of tris buffer, pH 7·4 (0·3 ml.), aconitase solution (0·1–0·2 ml.), neutralized inhibitor and water to 2·75 ml. was allowed to stand at room temperature for 10 min. Sodium (±)-*iso*citrate (0·5 μmole) was added and the reaction rate determined by measuring the extinction at 240 mμ from 0·5 to 4 min. after addition. Inhibition was determined by comparison with a control containing no inhibitor. Maximum inhibition under these conditions was 69% and inhibitions have been plotted as percentages of 69%. ○, Component 12, synthetic fluorocitric acid; ●, biosynthetic fluorocitric acid.

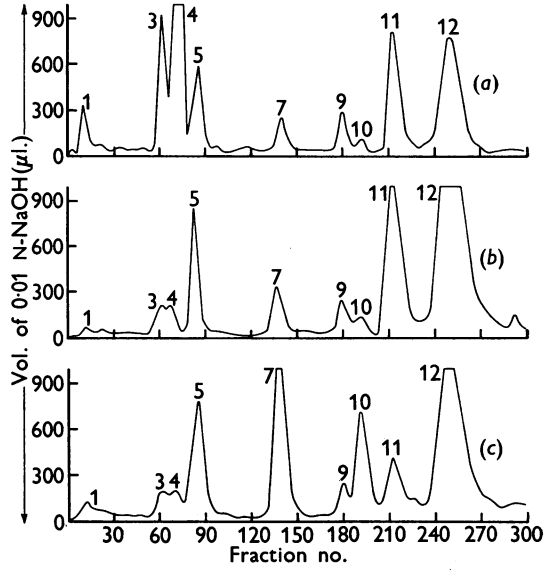

Fig. 4. Silicic acid-column chromatography of synthetic fluorocitric acid. Effect of treatment with excess of alkali: (*a*) untreated; (*b*) excess of 0·5N-NaOH at 20° for 24 hr.; (*c*) excess of 2N-NaOH at 60° for 1 hr. Fraction volumes were 2·0 ml. and eluting solvents were used as follows: fractions 1–49, butan-1-ol–chloroform (5:100, v/v); fractions 50–117, butan-1-ol–chloroform (15:100, v/v); fractions 118–168, butan-1-ol–chloroform (25:100, v/v); fractions 169–300, butan-1-ol–chloroform (35:100, v/v).

Table 3. *Effect of alkali on certain components produced by the silicic acid chromatography of synthetic trisodium fluorocitrate of low biochemical activity*

Samples (60 mg.) of trisodium fluorocitrate were treated as described in the Table, chromatographed on a silicic acid column and graphs were plotted of fraction number against quantity of 0·01N-NaOH required to neutralize the fractions. Figures represent the areas under the selected components expressed as a percentage of the total area under all the components eluted.

Treatment	Component 3	Component 4	Component 7	Component 10	Component 11	Component 12
Nil	11·0	22·2	3·9	1·3	13·6	23·9
0·1N-NaOH at 60° for 1 hr.	2·1	6·9	5·9	3·6	18·8	39·8
0·5N-NaOH at 20° for 24 hr.	2·2	2·2	4·7	2·0	18·0	46·2
0·5N-NaOH at 60° for 1 hr.	2·3	2·3	9·1	5·5	14·6	37·3
2N-NaOH at 20° for 24 hr.	2·6	2·6	12·6	7·4	9·8	33·8
2N-NaOH at 60° for 1 hr.	2·5	2·5	17·5	11·0	7·9	32·0

strong conditions were necessary: namely 0·1N-NaOH at 60° for 1 hr.

Optimum conditions for the alkaline hydrolysis of synthetic triethyl fluorocitrate. It appeared that the alkaline conditions previously used for the hydrolysis of triethyl fluorocitrate were too weak and that stronger conditions would give a better yield of the biochemically active component 12. With the results from Fig. 4 and Table 3 component 12 was obtained in maximum yield when for every mole of triethyl fluorocitrate not less than 6 g.-equiv. of NaOH at a concentration of 0·5N was used at 20° for 24 hr. Under these conditions analysis of the sodium fluorocitrate so formed produced a chromatographic pattern similar to that in Fig. 4 (*b*).

Acid hydrolysis of synthetic triethyl fluorocitrate. This was not studied so closely as alkaline hydrolysis, but the results were again complex. Under the conditions described by Rivett (1953), namely refluxing with 3N-HCl for 6 hr., column chromatography on silicic acid showed that components 3 and 4 were more prominent than under the optimum conditions for alkaline hydrolysis; also a large non-toxic component was always present around fraction 165. Components 11 and 12 were present in the same relative proportions as they were after alkaline hydrolysis. In order to separate the products of hydrolysis from HCl it was either necessary to extract them continuously with ether for about 10 hr. or to precipitate them as their barium salts with barium chloride. By this last method component 5 was not precipitated. It is therefore less convenient to separate the products of hydrolysis from the hydrolytic agent when HCl is used than when NaOH is used. Excess of NaOH may be readily removed by using a cation-exchange column as described.

Effect of acid on fluorocitric acid. Treatment of crude fluorocitric acid or its sodium salt with acid has little effect on the chromatographic pattern. When warmed with 0·5N-H_2SO_4 for 1 hr. at 65° the only significant change was the transformation of component 7 into one with its mid-point at fraction 85. These conditions would transform *cis*-aconitic acid into *trans*-aconitic acid and it was found that although *cis*-aconitic acid is some 13 fractions less mobile than component 7, treatment of the mixture of the two under the acid conditions described gave a single component at fraction 85.

Preparation of derivatives of synthetic fluorocitric acid components. Free fluorocitric acid as prepared from the crude synthetic specimens or those prepared biosynthetically was found to be extremely deliquescent. The majority of the chromatographically separated components were also highly deliquescent. Crystals have been obtained in a desiccator but outside it their life was but a few seconds. The sodium salts of the components were less deliquescent but could not be obtained in a crystalline form. The preparation of derivatives to help characterize the components have so far not been very successful. Attempts were made to prepare esters, amides, anilides, *p*-toluidides, *p*-nitrobenzyl esters, *p*-phenyl phenacyl esters and *S*-benzylthiouronium salts. The only crystalline compounds obtained were the ethyl ester of component 12 and the ethyl ester and amide of component 5. The difficulty in preparing crystalline derivatives was attributed to the presence of fluorine atoms in the molecules of the components, since similar derivatives were readily prepared with citric acid.

Estimation of citric acid in samples of fluorocitric acid. During the course of this work it became necessary to estimate the amount of citric acid present in specimens of fluorocitric acid. Some preparations of synthetic fluorocitric acid contained citric acid as an impurity and it was preferable to use citric acid-free preparations for further work. Specimens which were shown to be free of citric acid when estimated by the method of Pucher, Sherman & Vickery (1936) appeared to have an apparent citric acid content of up to 40% when estimated by the method of Taylor (1953). The difference between the estimations was traced to the addition of the reagents for oxidation and

bromination in Taylor's method whilst the specimen was still warm from its treatment with strong H_2SO_4. If oxidation and bromination are carried out immediately after treatment of the specimen with strong H_2SO_4 then the temperature of the solution is about 60° at the commencement of oxidation and bromination. This made no difference for citric acid itself, but it could apparently remove the fluorine from fluorocitric acid and so leave a product which was estimated as citric acid. It is evident that under some conditions the recommended cooling stage is necessary. All our preparations of biosynthetic and citric acid-free synthetic fluorocitric acid before chromatography gave a yellow colour when oxidation and bromination by Taylor's method were carried out in the warm. This test was also applied to several of the individual column-chromatographic components of citric acid-free synthetic fluorocitric acid under identical conditions, so that the temperature rise due to the dilution of the strong H_2SO_4 would be the same. Table 4 shows that a number of components gave a faint-yellow colour but components 11 and 12 gave it much more strongly. The intensity of the colour given by components 11 and 12 was also very similar.

Table 4. *Comparison of fluorocitric acid with citric acid in the method for the microdetermination of citric acid*

Sulphuric acid (27N; 5 ml.) was added to an aqueous solution of the sample (1 mg. of free acid in 5 ml. of N-H_2SO_4) and mixed. Precautions were taken to see that both the 27N-H_2SO_4 and the sample for every test were at the same (room) temperature. To the warm mixture was added immediately 5 ml. of an aqueous solution of 19·386 g. of KBr, 5·440 g. of $KBrO_3$ and 12·000 g. of NH_4VO_3 in 1 l.; after 20 min. 22·0% (w/v) $FeSO_4,7H_2O$ solution in N-H_2SO_4 (2 ml.) was added and the mixture left for 10 min. with occasional shaking to remove Br_2. Light petroleum (b.p. 80–100°) (6 ml.) was added and the mixture shaken for 1 min. The light-petroleum extract was removed, dried with anhydrous Na_2SO_4 and 5 ml. was shaken for 1 min. with 2% (w/v) Na_2S solution (3 ml.). The aqueous layer was removed and filtered into a Spekker 2 cm. microcell. The absorption was measured against a blank of N-H_2SO_4 from 15 to 30 min. after colour development; an Ilford 601 violet filter was used. Citric acid (100 μg. in 5 ml. of N-H_2SO_4) was used as a standard.

Sample	Weight (mg.)	*E*
Citric acid	0·1	0·69
Component 3	1·0	0·22
Component 4	1·0	0·15
Component 7	1·0	0·17
Component 9	1·0	0·13
Component 10	1·0	0·06
Component 11	1·0	0·91
Component 12	1·0	0·89

DISCUSSION

Silicic acid-column chromatography reveals the heterogeneous nature of all samples of fluorocitric acid. The many components present in synthetic samples may be grouped into two categories: those produced by side reactions in the synthesis and those produced by hydrolysis of the ester. A study of the synthesis of citric acid (Lawrence, 1897) would suggest that likely impurities due to side reactions in the synthesis of triethyl fluorocitrate would be ethyl oxalate, formed by decomposition of ethyl fluoro-oxaloacetate, ethyl fluorosuccinate, formed by the reduction of fluoro-oxaloacetate with zinc, and ethyl succinate, formed by the similar reduction of ethyl bromoacetate. In the synthesis in which ethyl bromo-[*Me*-^{14}C]-acetate was used, ethyl oxalate, ethyl fluoromalate or ethyl fluorosuccinate produced as impurities would not be radioactive. No sample chromatographed showed significant components of similar mobility to succinic acid or oxalic acid, nor did the prominent non-radioactive component 5 appear to be either fluoromalic acid or fluorosuccinic acid. The structure of component 5 is still being investigated.

The most interesting components produced during the chromatography of synthetic fluorocitric acid are 11 and 12 and they are probably chemically related. Since fluorocitric acid has two centres of asymmetry its synthesis would be expected to give two racemic diastereoisomers. It is very likely that chromatography on silicic acid columns would separate these and the similar chemical properties of components 11 and 12 suggest that they are the expected diastereoisomers. Component 12, (±)-fluorocitric acid, contains the enantiomorph that is inhibitory to aconitase and is the substance that blocks the tricarboxylic acid cycle in fluoroacetate-treated animals. Component 11, which may be designated (±)-*allo*fluorocitric acid, is not inhibitory to aconitase and contains the pair of enantiomorphs not synthesized by enzymic processes. The proportion of diastereoisomers formed in Rivett's (1953) method of synthesis would appear to be approximately 70% of (±)-fluorocitric acid and 30% of (±)-*allo*fluorocitric acid. Biosynthetic samples of fluorocitric acid when chromatographed on a silicic acid column contain no component equivalent to (±)-*allo*fluorocitric acid but they do contain one equivalent to the (±)-fluorocitric acid prepared by chemical synthesis. This component is about twice as active biochemically in biosynthetic as in synthetic samples. Thus it would appear that only one of the two enantiomorphs of (±)-fluorocitric acid is present in biosynthetic samples. This means that only one of the four possible optical isomers with the structure of

fluorocitric acid is formed *in vitro* by rabbit kidneys poisoned with fluoroacetate. The stereochemistry of fluorocitric acid is closely paralleled by that of *iso*citric acid, and whilst studying the synthesis of *iso*citric acid, Pucher & Vickery (1946) separated the two diastereoisomers of this acid. The similarity of the chemical properties of components 11 and 12 is shown by the hydrolysis of their esters. After mild alkaline hydrolysis of the synthetic ester the ratio of components 3 to 11 and 4 to 12 is large, but the ratios are progressively decreased as the pH and temperature of hydrolysis increases. This would suggest that components 3 and 4 are partial esters of (±)-*allo*fluorocitric acid and (±)-fluorocitric acid respectively. Equivalent-weight determinations show that components 3 and 4 are dibasic and hence may have one ester group intact. Simultaneously, with the removal of ester groups by hydrolysis, components 11 and 12 are degraded to form components 10 and 7 respectively. Another similarity between the chemical properties of components 11 and 12 is their extremely similar reaction under certain conditions in the estimation of citric acid by Taylor's (1953) method. The nature of components 10 and 7 is not yet clear, but the behaviour of component 7 after heating with dilute acid suggests that it may be *cis*-fluoroaconitic acid, and component 10 would then be the other structurally isomeric *cis*-fluoroaconitic acid.

For qualitative studies with aconitase the mixed hydrolysis products of the synthetic ester are often useful since only on one occasion was one of the by-products found to be inhibitory to aconitase. The presence of a biochemically active by-product must have been responsible for the observations by Morrison & Peters (1954) that synthetic fluorocitric acid caused a smaller decrease of citrate metabolism in guinea-pig-kidney particles but a greater inhibition of isolated aconitase than does biosynthetic fluorocitric acid. We now find that, as would be expected, component 12 or (±)-fluorocitric acid causes half as much inhibition both of citrate metabolism in guinea-pig-kidney particles and of isolated aconitase as does biosynthetic fluorocitric acid.

When synthetic fluorocitric acid is used as a quantitative biochemical inhibitor extreme caution will be necessary. It will be important to take into account impurities formed both during synthesis and hydrolysis of the ester and to make allowance for the biochemically inactive (±)-*allo*fluorocitric acid as well as the inactive enantiomorph of (±)-fluorocitric acid. If any method other than that of Rivett (1953) should be devised for the synthesis of fluorocitric acid, then the two racemic diastereoisomers would probably be formed in different proportions and due regard would have to be given to the different toxicity of any compound so produced. At present, with Rivett's method of synthesis of the ethyl ester, a sample of (±)-fluorocitric acid of constant properties and one that inhibits aconitase half as much as biosynthetic fluorocitric acid can be obtained only by separating it with a method such as silicic acid-column chromatography.

SUMMARY

1. Chromatography on paper and on ion-exchange and silicic acid columns has revealed the heterogeneous nature of five samples of fluorocitric acid.

2. Chromatographically pure samples of biosynthetic and synthetic fluorocitric acid have been prepared.

3. The biochemical activity of components of fluorocitric acid produced by silicic acid-column chromatography has been studied.

4. Two types of impurities have been observed in synthetic samples of fluorocitric acid: those produced during the synthesis of triethyl fluorocitrate and those produced during its hydrolysis.

5. The hydrolysis of triethyl fluorocitrate has been studied and optimum conditions have been devised for the production of (±)-fluorocitric acid.

6. The stereochemistry of fluorocitric acid and the separation of its diastereoisomers on a silicic acid column have been discussed.

7. A comparison has been made of the inhibition of aconitase both in guinea-pig-kidney particles and in an isolated state by purified biosynthetic and synthetic (±)-fluorocitric acid.

8. Attempts have been made to prepare derivatives of components of synthetic fluorocitric acid by methods which readily produce derivatives with citric acid.

9. The effect of components of synthetic fluorocitric acid on the quantitative estimation of citric acid has been studied.

We would like to thank Dr E. M. Gal, Dr N. F. Taylor, Dr J. Walker and Dr D. E. A. Rivett for samples of ethyl, sodium and barium fluorocitrate, and Miss P. Ivall, Mrs R. Jourdan, Mr B. Sloss and Mr G. Weston for technical assistance at various stages in the work.

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Estimation of Lactose in Rat Mammary-Gland Suspensions

By T. F. SLATER*
Department of Biochemistry, University College London, Gower Street, London, W.C. 1

(*Received* 16 *August* 1960)

Excised mammary tissue contains a variable quantity of milk or colostrum retained in the ducts and alveoli. The importance of correcting biochemical analyses of mammary tissue for this variable quantity of retained fluid has been repeatedly stressed (Folley & Greenbaum, 1947; Greenbaum & Slater, 1957*a*). The standard method of measuring the percentage of retained fluid in rat mammary-gland suspensions (Folley & Greenbaum, 1947) is based on the estimation of lactose in clear filtrates of mammary suspensions obtained after precipitation by ferric hydroxide, lactose being estimated by the chloramine-T method of Hinton & Macara (1927). The method of Folley & Greenbaum (1947) gives good recoveries of added lactose from mammary suspensions, and also compares well with other methods of lactose estimation when tried on samples of cow or rat milk (Slater, 1957). An alternative method for the estimation of lactose, based on heating with orcinol (Slater, 1957), is suitable for samples of milk or of mammary-tissue suspensions where lactose is the major carbohydrate present. The orcinol and chloramine-T methods do not agree when used with mammary-gland suspensions (Slater, 1957); the latter method gives higher lactose values. Although protein has been shown to affect chloramine-T values to a much greater extent than orcinol values (Slater, 1957) the discrepancy is not solely due to protein remaining in the ferric hydroxide filtrates.

This paper describes work done to elucidate the discrepancy between the methods which is particularly marked when the mammary tissue is taken from rats in late pregnancy (18–22 days), when the amount of lactose found by the chloramine-T method is approximately four times the value found by the orcinol procedure.

* Beit Memorial Fellow.

METHODS

The rats used were black and white adults of the Medical Research Council strain; body weight, 180–200 g. The length of pregnancy was assessed by examination of the foetuses. Animals were killed by cervical dislocation and the abdominal glands were quickly dissected and placed in ice-cold water; tissue suspensions were prepared with a top-drive blender (Folley & Watson, 1948) and were diluted 1:10 with cold water, Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951) with ox serum albumin as standard. Free α-amino acid groups were determined by Folin's method (Hawk, Oser & Summerson, 1947). Free sulphydryl groups were determined as described by Stadtman (1957). Deoxyribonucleic acid was estimated by the method of Greenbaum & Slater (1957*b*). Ferric hydroxide filtrates were obtained by diluting 5 ml. of the 1:10 suspension with about 15 ml. of water, adding colloidal ferric hydroxide drop by drop until coagulation was complete, making up to 25 ml. with water and filtering. Other methods of precipitation were by (*a*) trichloroacetic acid [equal volumes of suspensions and cold 10% (w/v) trichloroacetic acid were mixed, kept for 30 min. at room temperature, diluted and filtered]; (*b*) molybdophosphoric [equal volumes of suspension and 5% (w/v) molybdophosphoric acid were mixed, kept for 30 min. at room temperature, diluted and filtered]. Lactose was determined in such filtrates either by the method of Folley & Greenbaum (1947) or by the method of Slater (1957).

RESULTS AND DISCUSSION

The results of lactose estimations performed on ferric hydroxide filtrates are shown in Table 1. It can be seen that in late pregnancy the lactose titre given by the chloramine-T procedure is approximately four times the value given by the orcinol method. In lactation, however, the ratio is only 1·7. It would appear from these results that the chloramine-T procedure is being affected by some interfering substances with reducing properties